The occurrence and ecological role of plasmids in bacterial mycosphere dwellers

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Chapter 3

IncP-1β plasmids are important carriers of fitness traits for *Variovorax* species in the mycosphere – two novel plasmids, pHB44 and pBS64, with differential effects unveiled

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Abstract

The *Laccaria proxima* mycosphere strongly selects *Variovorax paradoxus* cells. Fifteen independent *V. paradoxus* strains, isolated from mycospheres sampled at two occasions, were investigated with respect to the occurrence of plasmids of sizes < 60-100 kb. Two *V. paradoxus* strains, HB44 and BS64, were found to contain such plasmids, which were coined pHB44 and pBS64. Replicon typing using a suite of plasmid-specific PCR systems indicated that both plasmids belong to the IncP-1β group. Also, both were able to mobilize selectable IncQ group plasmids into *Escherichia coli* as well as *Pseudomonas fluorescens*. Moreover, they showed stable replication in these organisms, confirming their broad host range. Strain BS64 was cured of pBS64 and plasmid pHB44 was subsequently moved into this cured strain by making use of the IncQ group tracer plasmid pSUP104, which was then removed at elevated temperature. Thus, both plasmids could be screened for their ability to confer a phenotype upon strain BS64. No evidence for the presence of xenobiotic degradation and/or antibiotic or heavy metal resistances was found for either of the two plasmids. Remarkably, both could stimulate the production of biofilm material by strain BS64. Also, the population densities of pBS64-containing strain BS64 were temporarily raised in liquid as well as soil systems (versus the plasmid-cured strain), both in the presence of the fungal host *Lyophyllum* sp. strain Karsten. Strikingly, plasmid pHB44 significantly enhanced the fitness of strain BS64 in soil containing *Lyophyllum* sp. strain Karsten, but decreased its fitness in soil supplemented with extra $FeCl_3$. The effect was noted both in separate (no inter-strain competition) and joint (competition) inoculations.

Introduction

Most soils are teeming with bacterial life. Soils also contain organic matter in different amounts and constitution. For an average (heterotrophic) soil bacterium, the available sources of carbon and energy are actually quite scarce, as soil organic matter is normally complex and poorly available (Standing and Killham, 2007). Hence, it is posited that many bacteria in soil are selected for fitness on the basis of their capacities to acquire, and thrive on, sources of carbon and energy beyond what is present as (recalcitrant) soil organic matter. Such sources are often heterogeneously distributed in space and time, and so-called hot spots with enhanced carbon availability have been found to exist in soil. One such hot spot is constituted by the mycosphere, i.e. the sphere of influence of the dense bundle of hyphae that emerges from soil to form fungal fruiting bodies (Warmink and van Elsas, 2008). A range of soil fungi, e.g. ectomycorrhizae, arbuscular mycorrhizae and/or saprotrophic fungi produce such bodies (Finlay, 2007). Given the heterogeneity of soil in space and time, soil bacteria are likely driven by the ability to quickly adapt to the conditions (in terms of available substrate and physicochemistry) that emerge locally in hot spots such as the mycosphere. Hence, the fitness of many bacteria in soil is most likely determined by both their intrinsic capacities and their flexibilities to colonize new niches, boiling down to their efficiency in acquiring and utilizing newly emerging substrates.

The adaptability of bacteria in soil may thus be well served by horizontal gene transfer (HGT), as beneficial traits present in the so-called horizontal gene pool may readily enter bacterial cells and be fixed in them (Thomas and Nielsen, 2005). Plasmids are major contributors to the dissemination of genes in this horizontal gene pool (Gogarten and Townsend, 2005; Thomas and Nielsen, 2005). In particular plasmids with broad host range offer genetic flexibility to the bacterial community at large, possibly allowing genetic interconnections between species, genera, families or even kingdoms (Thomas, 2000; Thomas and Nielsen, 2005). A key group of broad-host-range plasmids with increasing environmental relevance is the IncP-1 group (Sota and Top, 2008). Plasmids belonging to this group have been isolated from environments like wastewater treatment plants, hospital effluents, river sediment, seawater and even contaminated soils (Daane et al., 2001; Heuer et al., 2004; Rhodes et al., 2004; Schluter et al., 2007). Such plasmids have been shown to be able to reside in a wide range of (Proteo)bacteria, revealing remarkable transfer abilities to various recipients (Dennis, 2005; Schluter et al., 2007; Szpirer et al., 1999). The IncP-1 plasmid group has been divided into six subgroups (denoted α through ζ), across which the IncP-1α and

IncP-1β groups have been described most extensively (Norberg et al., 2011). Across all hitherto-found IncP-1 plasmids, fairly conserved backbones have been found, which are interspersed with accessory genes that are integrated at three typical insertional hotspot sites (Boersma, 2010; Heuer et al., 2004; Norberg et al., 2011).

In a previous study, we showed that *V. paradoxus* related organisms are specifically selected in the mycosphere of the ectomycorrhizal (EM) fungus *Laccaria proxima* in the field (Boersma et al., 2010). More precisely, organisms of this type prevail among the culturable bacterial community of the mycosphere of *L. proxima*, as respectively 9.0 and 7.3 % of the communities that were cultured from the hyphal bundles in sampling years 2004 and 2006 (versus numbers below 1% in corresponding bulk soils). A representative strain of this group, denoted HB44, was analyzed with respect to its ecological (mycosphere) competence. Strain HB44 was shown to be able to grow at the expense of compounds, in particular glycerol and acetate, that were released by fungal hyphae as the sole carbon sources (Boersma et al., 2010). Using a PCR-based screen on colony material (targeting the *vir*B4 gene), strain HB44 was indicated to contain a type IV secretion system (T4SS) (Boersma, 2010). Given the fact that T4SSs are often located on plasmids, encoding self-transfer and gene mobilizing systems, we hypothesized that strain HB44 might contain a plasmid. Such a plasmid might enable it to cope with the conditions of soil or a developing mycosphere.

In the light of the initial finding of evidence for the presence of a T4SS in *V. paradoxus* strain HB44, we examined this strain, next to 14 other *V. paradoxus* strains, for the presence of plasmids. Here, we describe the prevalence, characteristics, phenotype, transfer and mobilization capacity, preliminary sequence analysis and putative ecological role of the plasmids found in the investigated strains. To the best of our knowledge, this is the first detailed analysis of IncP-1β plasmids in the context of their potential ecological role in the mycosphere.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The mycosphere-isolated *V. paradoxus* strains, including strains HB44 and BS64 (isolated in 2004 and 2006), were grown in either R2A or Luria-Bertani (LB) broth (tryptone 10 g, yeast extract

5 g, NaCl 5 g, distilled water 1 L; pH 7.2) at 28 °C for 24 h. A streptomycin-resistant (Sm^r) mutant of strain BS64 with growth rate similar to the parent was selected and stored at -80°C in 20% glycerol. The *Escherichia coli* strains used for mating experiments were grown overnight at 37 °C in LB broth containing the appropriate antibiotics (Table 1). *Pseudomonas fluorescens* R2f was grown overnight in LB broth at 28 °C. Agar (1.75%) was added to the media when necessary. Antibiotics were added as appropriate and their concentrations can be found in Table 1.

Table 1. Strains and plasmids used

Strain	Antibiotic resistance	Plasmid / Inc group
Variovarax paradoxus		
HB44 (pHB44)	-	pHB44 / IncP-1β
BS64 (pBS64)	-	pBS64 / IncP-1β
BS64 Sm^r	Sm^r	-
BS64 (pHB44)	-	pHB44 / IncP-1β
Escherichia coli		
JM101 (RP4)	Ap^r, Tc^r, Km^r	RP4 / IncP-1α
K12 (R751)	Tmp^r	R751 / IncP-1β
JM109 (pTP6)	-	pTP6 / IncP-1β
MC1061 (pKJK5)	Tc^r	pKJK5 / IncP-1ε
CSH52 (pSUP104)	Tc^r Cm^r	pSUP104 / IncQ
HB101(pMOL187)	Km^r Gm^r	pMOL187 / IncQ
CV601Rp^r	Rp^r	-
CV601 Rp^r (pHB44; pMOL187)	Rp^r Km^r Gm^r	pHB44; pMOL187
CV601 (pIPO2)	Tc^r	pIPO2/PromA
Pseudomonas fluorescens		
R2f Rp^r	Rp^r	-

Antibiotics and concentration used (in ug/ml): Sm -streptomycin 50; Tc -tetracycline 25; Cm -chloramphenicol 25; Rp - rifampicin 100; Km - kanamycin 50; Gm - gentamycin 50; Ap-ampicillin 50; trimethoprim 10.

-: indicates no resistance is present

IncQ plasmid mobilization and self-transfer of putative plasmids to E. coli CV601 and P. fluorescens R2f

Plasmids initially found in *V. paradoxus* strains HB44 and BS64 – denoted pHB44 and pBS64 – were transferred to *E. coli* CV601 (resistant to rifampicin - Rp^r) as well as *P. fluorescens* R2f Rp^r using triparental matings (Mela et al., 2008). Mobilizable plasmids pMOL187 (equipped with kanamycin (Km) and gentamycin (Gm) resistances) or pSUP104 (chloramphenicol (Cm) and tetracycline (Tc) resistances) served as the tracer plasmids. Thus,

V. paradoxus HB44 (pHB44), as well as BS64 (pBS64) served as donors, *E. coli* HB101 (pMOL187) or *E. coli* CSH52 (pSUP104) as helper strains and *E. coli* CV601 Rp^r or *P. fluorescens* R2f Rp^r as the recipients. Selected transconjugants were investigated as to the co-presence of plasmids pHB44 and/or pBS64 using plasmid extraction and agarose gel detection, in addition to PCR-based replicon typing. Transfer frequencies (transconjugant per recipient ratio's) of these matings were compared to those of triparental matings with *E. coli* JM109 (RP4) as the donor. Confirmed transconjugant colonies were selected, purified and stored after growth to a fully-grown culture (-80°C, 20% glycerol).

Plasmid stability and curing

The stabilities of the novel plasmids pHB44 and pBS64 in their original hosts as well as in *E. coli* CV601 were determined by culturing the plasmid-containing strains (starting inoculum about 10^5 cells ml^{-1}) at, respectively, 28 or 37 ^oC in LB broth, using sequential batch cultures. Seven subsequent 1:1000 transfers were thus performed, allowing up to about 70 generations. The cultures of each batch were checked for the loss of plasmids using plating to single colonies, followed by colony PCR targeting the *trf*A gene as a proxy for the presence of an IncP-1β plasmid (see further; Gotz et al., 1996). *trf*A-negative single colonies were subjected to plasmid isolation to confirm the absence of plasmid DNA.

To produce plasmid-cured derivatives of *V. paradoxus* strains HB44 (pHB44) and BS64 (pBS64), we applied serial batch transfers of the relevant cultures using (1) raised temperatures (33º and 37º C) and (2) sub-inhibitory concentrations of novobiocin (7 µg/ml) and ethidium bromide (4 µg/ml). After each transfer, in particular focusing on transfers 5, 10 and 20, 20-50 colonies were checked per culture by colony PCR (on the basis of the *trf*A gene), to assess the putative loss of the IncP-1β plasmid. Potentially cured clones were subjected to plasmid extractions in order to reveal the physical absence of a plasmid band.

Plasmid extraction and molecular sizing

Plasmid DNA was extracted following a (slightly modified) extraction protocol (Birnboim and Doly, 1979). The final DNA pellets were resuspended in TE buffer (Sambrook et al., 2004). Plasmid DNA was visualized on 1% agarose gels using ethidium bromide staining. Molecular sizing of plasmid bands was performed by comparing the extracted covalently closed circular (ccc) DNAs with ccc DNA of a range of plasmids of known size, i.e. pMOL187 (20 kb), pIPO2T (45 kb), R751 (53 kb) and RP4 (60 kb) (Table 1).

Replicon typing and restriction analysis of plasmid DNA

We used direct PCR-based plasmid replicon typing to infer the incompatibility group of the (broad-host range) plasmids found. This was performed using primer sets targeting the incompatibility groups IncQ, IncW, IncP-1, IncN and IncA/C, as previously described (Gotz et al., 1996).

Digestion of plasmids pHB44 and pBS64 was performed as follows (Sambrook et al., 2004): a 100 µL DNA digestion mix consisted of 10 µL digestion buffer, 4 µL enzyme, 100 µg DNA. Sterile water was added to an end volume of 100 µL. Digestion was done for up to 1 (using *EcoR*I and *Sph*I) or 2 h (*Bam*H1 and *Hind*III) at 37°C. As controls, these enzymes were also used to digest the reference plasmids R751, RP4, pIPO2T and pSUP104.

Hybridization analysis and initial shotgun sequencing to identify plasmid pHB44

Together with plasmids RP4 (IncP-1α), pKJK5 (IncP-1ε), R751 (IncP-1β) and pTP6 (IncP-1β) as controls, properly-restricted plasmid DNA obtained from strains HB44 (pHB44) and BS64 (pBS64) was subjected to gel electrophoresis followed by Southern hybridization with a PCR-generated *trf*A probe characterizing the incompatibility (Inc) type, as described (Smalla et al., 2000). For initial sequencing, we focused on plasmid pHB44. Plasmid DNA was extracted from *E. coli* (pHB44; pMOL187) and purified by gel electrophoresis. Bands containing the pHB44 ccc DNA were collected by excision from gel and purified (Sambrook et al., 2004), after which they were subjected to commercial sequencing using Roche 454 pyrosequencing technology. Following extensive filtering of the about 400-bp sequence reads (based on quality, presumptive plasmid localization, and fold), a total of about 1,600 reads were obtained, which matched the canonical IncP-1β plasmid backbone in about 12 contigs (Boersma, 2010).

Screening for traits conferred by plasmids pHB44 and pBS64 upon V. paradoxus strain BS64

Minimal inhibitory concentrations (MICs) of selected antibiotics for *V. paradoxus* strains BS64 (pBS64), plasmid-less BS64 and BS64 (pHB44) were assessed by dilution plating on R2A agar supplemented with ampicillin (Ap), kanamycin (Km), chloramphenicol (Cm), nalidixic acid (Nx), streptomycin (Sm), colistin sulphate (CS), nitrofurantoin (Nf), tetracycline (Tc), penicillin (Pc), spectinomycin (Sp), vancomycin (Vm),ergebicillin (Eb) and erythromycin (Em) (0, 5, 10, 20, 50, 100 µg/ml). MICs for heavy metals were assayed by dilution plating on LB agar supplemented with zero-1-2-5-10-25-100 µg/ml of $HgCl_2$ or zero-

4-8-16-32-64-128-256 μg/ml of (in separate) $CuSO_4$, $NiCl_2$, $CoCl_2$, $ZnSO_4$ and $CdCl_2$. Plates were incubated for up to 7 days at 28°C and checked daily for the growth of single colonies.

To assess growth on xenobiotic compounds, all three aforementioned strains were grown (with shaking) in flasks containing 20 ml M9 medium (Sambrook et al., 2004) supplemented with 0.5% of 2-chlorobenzoic acid, 3-chlorobenzoic acid, 4-chlorobenzoic acid, chloro catechol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenoxy acetic acid, chloro acetate (sodium form), salicylate, toluene, xylene and 4-methyl toluene sulfonate as sole carbon sources. Negative controls consisted of M9 without an added C source and positive ones of M9+0.5 % glucose. Flasks were incubated at 28°C and OD measurements as well as dilution platings were performed to detect the growth daily for up to 7 days. A summary of assessments of phenotypes conferred by plasmid pHB44 and pBS64 can be found in Table 3.

Assessment of plasmid-induced biofilm formation

Biofilm formation by *V. paradoxus* strains BS64 (pBS64), BS64 (pHB44) and plasmid-less BS64 was tested in replicate 96-well microtiter plates (Costar, Corning, USA) using different incubation times, i.e. 12,18 and 24 h (Djordjevic et al., 2002). Early stationary-phase cells were washed, adjusted to the same OD values (using adsorption at 600 nm) and added to the microtiter plate wells, after which plates were incubated. At each time point, the supernatant was removed and the attached biofilm cells were washed with water. This was followed by addition of 0.5% crystal violet (CV) and incubation for 10 min. After washing, the crystal violet was extracted from the biofilm by dimethyl sulfoxide (DMSO) followed by absorbance measurement at 575 nm. The efficiency of biofilm formation was determined by normalizing the crystal violet signal (575 nm) with the cell signal (600 nm), as described in (Djordjevic et al., 2002).

Dynamics and utilization of compounds released by L. sp. strain Karsten

To examine the ability of *V. paradoxus* strains BS64 (pBS64), BS64 (pHB44) and plasmid-less BS64 to utilize fungal-released compounds as carbon sources, spent *L.* sp. strain Karsten L9+propionate growth medium (pH 6.5) was used as described (Boersma et al., 2010). The fungus was first grown in triplicate 250-ml Erlenmeyer flasks containing 50 ml L9 supplemented with 1% propionate as the sole carbon source (pH 4.5). Propionate was chosen, because strain BS64 could not metabolize this compound. After 14 d of fungal growth,

supernatants were harvested using centrifugation and sterilized by filtration (0.22 mm). Freshly-grown cells of all three strains were then added to triplicate supernatants at 10^5 cells per ml, after which flasks were incubated (28°C, shaking 200 rpm). As controls, the strains were tested in unused L9+propionate medium. Bacterial growth was followed by OD540 measurements, dilution plating and CFU counting on days 1, 4, 6, 8 and 14.

To further examine potential effects of the plasmids, another *L.* sp. strain Karsten infested medium was used in 100 ml bottles, as described previously (Nazir et al., 2013). It consisted of 0.5 g potato flakes, 1 yeast tablet (Pax), 2 g light malt extract, 0.5 g dextrose, 200 µL Hesi pk 13/14 and 100 mL sterile demineralised (MilliQ) water. *L.* sp. strain Karsten, following its growth on oat flake agar (OFA; consisting of 30 g oat flake and 15 g agar in 1 L of water [Warmink, 2009]) for 3-4 days, was added as 1x1cm blocks of fungal mycelium and then allowed to grow out at 20°C until a mycelial mat had formed at the liquid-air interface (30-45 d). Then, freshly-grown and washed cells of strains BS64 (pBS64), BS64 (pHB44) and plasmid-less BS64 were added, in quadruplicate, at 10^5 cells per ml. Bottles were incubated still at room temperature. Samples of the liquid were taken biweekly and dilutions plated on R2A plates. After incubation (24°C, >= 2 d), CFU counts were determined. Moreover, we scored the primordia set at the fungal mats, as described previously (Nazir et al., 2013).

Soil microcosms and population dynamics in soil with L. sp. strain Karsten mycelium as related to the level of Fe

Fresh soil from a field in Gieterveen, the Netherlands (denoted G soil), was used throughout. The soil was subjected to autoclaving at least twice until sterile. Soil sterility was confirmed by the absence of bacterial colonies or fungi on LB or PDA agar plates, respectively (incubated for 1-5 days at 28^0C) that had been inoculated with soil extracts. The sterilized soil had 2% organic matter, was moderately acid (pH-KCl 4.9) and contained an estimated 4 µM of Fe per g dry soil. Soil microcosms were constructed and inoculated with *Lyophyllum* sp. strain Karsten as described (Warmink, 2009). Briefly, using a triparted Petri dish, one compartment was filled with OFA (Warmink, 2009). The two other compartments were filled, at bulk density 1.2, with sterile G soil amended with 0.5% $CaCO_3$ to establish a soil pH of 6.8. To investigate whether the presence of a plasmid could enhance the competitiveness of strain BS64 for Fe in the mycosphere, two types of soil were used, one without additional Fe^{3+} and one with additional Fe^{3+} (as $FeCl_3$) at a concentration of 5 µM $FeCl_3$ over the water content of the soil (75% of WHC is 15% total water content; [Warmink,

2009]). Following inoculation with the fungus, the Petri dishes were sealed with parafilm to stabilize moisture contents and incubated at 28 °C for 5 days, at which time the fungal front had entered around 10 mm in both soil compartments.

Population dynamics in separate inoculation experiments - Freshly-grown and washed cells of *V. paradoxus* strains BS64 (pBS64), BS64 (pHB44) and plasmid-less BS64 were introduced (100 µl) - in triplicate - into the soil at/under the fungal mat, at about 10^4 cells g^{-1} soil. Control plates did not receive fungal inoculum. The microcosms were sealed and incubated at 28^0C. Over time, (4-mm dia core) samples were taken at the inoculation sites and processed for dilution plating. The bacterial inoculant densities were then monitored by CFU counting as in the previous section.

Population dynamics in joint inoculation experiments - This experiment addressed the competitive behavior of plasmid-containing and plasmid-less cells using joint inocula of *V. paradoxus* BS64 (pBS64)/ BS64 Sm^r as well as BS64 (pHB44) / BS64 Sm^r, at about 10^5 cells g^{-1} soil. At each sampling, CFU counts were obtained from Sm-containing and from non-antibiotic agar media, and the densities of each member of the pair were thus determined using direct counts on the former (strain BS64 Sm^r) and on the latter medium. Subtraction was used to infer the density of the plasmid-containing strains. Randomly-picked colonies on the streptomycin-containing plates (n=20 per replicate system) were checked for the putative presence of plasmids using *trf*A-based colony PCR. Plasmids were not detected on Sm-containing plates in any of the treatments, and so plasmid transfer was estimated not to bias our observations concerning competitive behavior of the respective strains.

Statistical analysis of the data

All experiments were performed in triplicate per treatment. Several experiments were repeated in time. At each time point, the data obtained were log-transformed, after which average values and standard deviations were determined. The assumption of normality was tested with Shapiro–Wilk statistics and differences between treatments were tested for significance with One-Way ANOVA and Tukey's HSD test ($P < 0.05$), in the statistical program PAST (Hammer et al., 2001). Bars in graphs and the number between brackets behind means of values represent the standard errors of the mean (SE).

Data availability

All data supporting this publication have been stored in the University of Groningen localized CEES data wiki (www.rug.nl) and are available freely upon request to: ceesdata@rug.nl.

Results

Detection and preliminary identification of plasmids across fifteen V. paradoxus strains.

Thirteen out of 15 *V. paradoxus* like strains obtained from the *L. proxima* mycosphere (six from a 2006 sample and nine from a 2004 sample [Boersma et al., 2010]), did not reveal any vestige of (small, i.e. < 100 kb) plasmid DNA following plasmid extractions. The two remaining strains, HB44 and BS64, did indeed harbor small-sized plasmids, which are, from here on, coined pHB44 and pBS64 respectively. Molecular sizing revealed plasmid pHB44 to be about 60 kb and plasmid pBS64 about 65 kb in size. This was corroborated by analysis of the restriction fragments generated by *Sph*1 and *EcoR*1 digestions, which specified the size of pHB44 as 60.0 and that of pBS64 as 65.5 kb.

For reasons of clarity, we will, from here on, provide the plasmid name after the strain name. Replicon typing (Gotz et al., 1996) of strains HB44 (pHB44) and BS64 (pBS64) yielded clear 241-bp amplicons with the IncP-1β specific *trf*A system, whereas no such amplicons were generated with the remaining 13 strains. Comparative Southern hybridization of plasmid pHB44 DNA (restriction using *Xba*1) with a PCR-generated *trf*A probe revealed the presence of single bands at positions coinciding with bands generated for the typical IncP-1β plasmids R751 and pTP6, away from bands generated on the IncP-1α plasmid RP4 and the IncP-1ε plasmid pKJK5. Sequencing of the 241-bp *trf*A amplicons of the two plasmids then revealed these to be indistinguishable from the available sequence of plasmid R751, and different from the sequences of any of the other IncP-1 plasmids (Fig.1). Thus, on the basis of this combined evidence, plasmids pHB44 and pBS64 reveal features of IncP-1β plasmids. A preliminary account of the plasmid pHB44 genome sequence, obtained from shotgun sequencing of *E. coli* CV601 (pHB44; pMOL187) extracted plasmid-enriched DNA (see later) revealed that many genes of the canonical IncP-1β plasmid backbone are present (44 in total identified). In this, a central control region, a plasmid transfer region and a plasmid maintenance region could be distinguished (Table 2). In addition, several accessory genes, mostly with potential function in membrane-bound processes, could be determined (Table 2).

In particular the presence of a gene that might confer an enhanced capacity to acquire Fe from the external milieu (putative *ton*B receptor involved in Fe uptake) was remarkable.

Table 2 – List of putative genes found on plasmid pHB44 by shotgun sequencing of DNA from *Escherichia coli* CV601 (pHB44; pMOL187)

Predicted genes*	Predicted function**	Explanation and remarks**
Conjugative transfer (*tra*) region		
*tra*C	Initiation of rolling-circle replication for transfer	
*tra*D	DNA 'processing' protein	Details in (Schluter et al., 2007)
*tra*E	DNA topoisomerase	
*tra*F	Signal peptidase	Serine protease that processes and cyclizes subunits of T4SS pilus
*tra*G	Coupling protein	Belongs to the VirD4 family of the T4SS. Interacts with the cytoplasmatic face of the T4SS and guides the 'relaxosome' complex to mating channel
*tra*H	Protein involved in 'relaxosome' stabilisation	Component of 'relaxosome' complex assembled at the origin of transfer (*ori*T)
*tra*I	DNA relaxase	Involved in catalysing single-strand nicking at the *nic* site within *ori*T
*tra*J	Protein that interacts with origin of transfer (*ori*T)	Component of 'relaxosome' complex assembled at the *ori*T
*tra*K	Second protein that interacts with *ori*T	
*tra*L/ *tra*M	Function unknown, but involved in conjugative transfer	
Mating-pair-formation (*trb*) region		
*trb*A	Transcriptional regulator of mating pair formation (mpf) and conjugative transfer	Controls expression of the *tra* and *trb* operons
*trb*B	ATPase in mpf	Energizes DNA transport process
*trb*C	Precursor of the major pilus subunit	Structural protein of mating system
*trb*D	Function unknown, probably structural	
*trb*E	ATPase in mpf	T4SS ATPase, VirB4 family, biogenesis of the secretion system by hydrolysis of NTPs
*trb*F	Minor pilus subunit or chaperone involved in pilus assembly	Involved in structure of T4SS
*trb*G	Probable effector translocator	T4SS VirB9/CagX family
*trb*H/*trb*I	Function unknown	
*trb*J/*trb*K	Entry exclusion	Prevents uptake of IncP-1 plasmid into a cell which already harbors an IncP-1 plasmid
*trb*L/*trb*M	Function unknown	
*trb*N	Transglycosylase predicted to be involved in lysing peptidoglycan	Catalysis of lysis of the peptidoglycan layer at the secretion apparatus assembling site
*trb*O/*trb*P	Function unknown	
upf 30.5	Outer membrane protein	Role in mating pair formation has to be proven
upf 31.0	Major outer membrane lipoprotein	Possibly involved in DNA methylation
Replication region		
*trf*A	Replication initiation protein	Typical for the IncP-1 plasmid replication machinery
ssb	Single-strand DNA binding protein	Involved in vegetative plasmid replication
Central control, stability and maintenance regions		

*kor*B	Transcriptional repressor involved in controlling of plasmid partitioning	DNA-binding protein that recognizes a *cis*-acting site of the plasmid and is involved in active control of partitioning of plasmid copies into daughter cells during cell division
*inc*C1/ *inc*C2	Processing of NTPases, interaction with KorB, energization of the partitioning process through NTP hydrolysis	Involved in active partitioning
*kor*A	Transcriptional repressor involved in controlling of plasmid partitioning	DNA-binding protein involved in transcriptional regulation
*kle*F/*kle*E/ *kle*B/*kle*A	Modulation of the efficiency of active partitioning	Regulators of stable maintenance mechanism. *kle*E: transmembrane protein; *kle*B: zinc-binding moiety
*kor*C	Transcriptional repressor involved in control of plasmid partitioning	DNA binding protein involved in transcriptional regulation
*klc*B	Stable inheritance protein	Modulate the efficiency of active partitioning
*klc*A	Anti-restriction protein	
*klu*A	Addiction module antitoxin protein, transcriptional regulator	Involved in putative postsegregational killing
*klu*B	Addiction module toxin protein, plasmid stabilization system protein	
Accessory gene region		
*ton*B	Putative membranous Fe receptor	putative Fe uptake system
*mfs*1	Transporters of MFS1 family	putative Fe uptake system

*all predicted genes matched genes of (broad host range) IncP-1β plasmid R751 (99-100% nucleotide sequence similarity)
** Predicted function: data derived from plasmid R751 and from (Schluter et al., 2007)
Explanation: T4SS= type IV secretion system (involved in DNA transfer)

Mobilization of an IncQ plasmid and transfer of plasmids pHB44 and pBS64 to E. coli and P. fluorescens

IncQ plasmid mobilization experiments were performed with *V. paradoxus* strains HB44 (pHB44) and BS64 (pBS64) as putative mobilizer/donor strains. In the two systems, transconjugant colonies (indicating the presence of the IncQ plasmid) were detected with both the *E. coli* and *P. fluorescens* recipients. About 20 (*E. coli*) and 100% (*P. fluorescens*) of randomly-picked transconjugant colonies also carried plasmids pHB44 or pBS64. In the RP4-based control (with frequency of mobilization at about 1.2 x 10^{-2} per recipient), these frequencies were about 10%. Thus, the transfer frequencies for pHB44 and pBS64 were on the order 10^{-4} to 10^{-5}, or about 10- to 100-fold lower than that of the RP4-driven transfer ($1.2x10^{-3}$). Plasmids pHB44 and pBS64 clearly interconnect *V. paradoxus* (β-Proteobacteria) with *E. coli* and *P. fluorescens* (γ-Proteobacteria), confirming the broad-host-range character of both novel IncP-1β type plasmids.

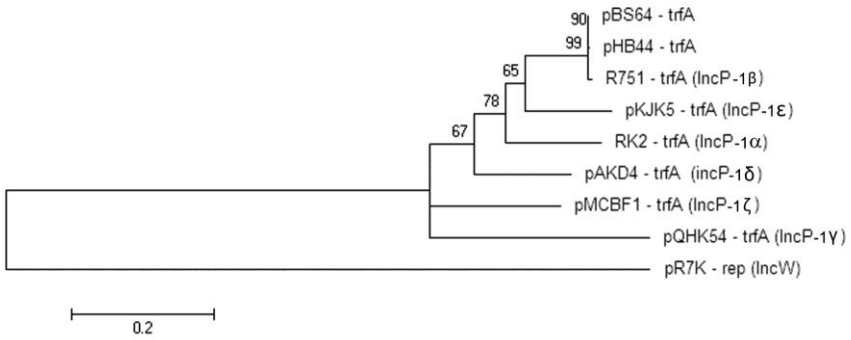

Fig. 1 Rooted maximum parsimony based tree of the trfA gene showing the affiliation of two novel V. paradoxus plasmids, pHB44 and pBS64, with the IncP-1β class of plasmids. Reference plasmid sequences were obtained from the NCBI database. Origins of the reference plasmids: R751, Enterobacter aerogenes; pRK2, Pseudomonas aeruginosa RK2; pR7K, Providencia rettgeri R7K; pKJK5, pAKD4, pMCBF1, pQHK54,uncultured bacteria from environmental samples

Stability of plasmids pHB44 and pBS64 in their hosts and curing

Plasmids pHB44 and pBS64 were quite stable in their original hosts *V. paradoxus* HB44 (pHB44) and BS64 (pBS64). Even after ~100 generations, no plasmid loss was found. In contrast, plasmid pHB44 in *E. coli* CV601 revealed an initial phase of plasmid stability followed by a phase of severe instability. After approximately 20 generations, the majority (87%) of the tested colonies still harbored plasmid pHB44, but after 30 generations this percentage had dropped to below 2, after which it remained at this low level.

All six plasmid-curing experiments performed with strain HB44 (pHB44) (sampled after 35, 70 and 100 generations) failed to yield plasmid-cured derivatives. In contrast, we did produce a pBS64-cured derivative of strain BS64, after about 6 transfers (60 generations) under growth-limiting (elevated temperature) conditions. Several colonies that were tested using the *trf*A-based PCR system as well as plasmid extraction showed convincing evidence for the loss of plasmid pBS64 from strain BS64. Two clones of the plasmid-less strain BS64 were stored and tested for growth rate in LB broth (batch culture) versus the plasmid-containing wild-type. Using OD measurements, we could not discern any growth advantage or disadvantage of the plasmid-cured strain versus the wild-type ($P>0.05$). We thus deemed the plasmid-less strain BS64 to be suitable for further ecological experimentation with respect to competitiveness and ecological behavior versus plasmid-containing strain BS64.

Introduction of plasmid pHB44 into plasmid-cured strain BS64

To assemble a suite of strains for plasmid-encoded phenotype testing, we transferred plasmid pHB44 into the plasmid-cured *V. paradoxus* strain BS64, with the help of the IncQ tracer plasmid pSUP104, which was subsequently removed successfully by using elevated temperature (60 generations) (data not shown). This yielded *V. paradoxus* strain BS64 (pHB44), which revealed a growth rate in LB broth (batch culture growth) roughly similar to that of strains BS64 (plasmid-less) and BS64 (pBS64), indicating it was 'fit' enough for experiments on phenotype. The comparative use of the *V. paradoxus* strains BS64 (pBS64), BS64 (pHB44), and plasmid-less BS64, next to HB44 (pHB44), allowed us to make inferences about the effects exerted by the two plasmids on the phenotype of strain BS64, as below.

Phenotypic characterization – antibiotic / heavy metal resistances and biodegradative capacities

Extensive screenings for antibiotic and heavy metal resistances and xenobiotic-degradative capacities showed that the presence of plasmids pHB44 and pBS64 in strain BS64 did not add any conspicuous extra trait that could be expressed in this host. The same was found for the *E. coli* transconjugants. Specifically, no resistances to Cm, CS, Km, Nx, Sm, Tc, Em and Sp were found, whereas resistances to Ap, Pc, Nf and Vm were found in both the plasmid-containing and plasmidless strains. Moreover, we found no evidence for any effect of the two plasmids in *V. paradoxus* BS64 on heavy metal resistance. The MIC values were 0.5 (Hg), 16 (Co), 256 (Cu, Zn and Ni) and 16 (Cd). For the latter four metals, there was a striking difference with the MIC values of strain HB44 (pHB44), which were 16 (Cu, Zn), 32 (Ni) and 8 (Cd). Putative induction of Tc and Hg resistances by pre-incubation on low concentrations of these compounds yielded equally negative findings.

Finally, plasmids pHB44 and pBS64 did not confer the capability to degrade any of the tested organic C compounds on the *V. paradoxus* BS64 host, as both the plasmid-containing and plasmid-less strains showed an absence of growth with these potential carbon and energy sources. Moreover, strain HB44 (pHB44) also did not degrade any of the tested compounds.

Effect of plasmids pHB44 and pBS64 on the capacity of V. paradoxus BS64 to form biofilms

Plasmids pHB44 and pBS64 did affect biofilm formation by *V. paradoxus* BS64, as evidenced from the microtiter plate method (Fig. 2). After 12h, clear differences in the

amount of biofilm formed by the two plasmid-harboring strains versus the plasmid-less (control) strain were observed, as the biofilms produced by strains BS64 (pBS64) and BS64 (pHB44) were significantly larger ($F = 14.0$, $P < 0.001$) than those produced by plasmid-less strain BS64 (Fig. 2A). After 18 h, this effect was still present ($F = 16.0$, $P < 0.001$), with strain BS64 (pBS64) showing the highest amount of biofilm, followed by BS64 (pHB44) and the control BS64 (Fig. 2B). Finally, at 24 h of incubation, the trend was even stronger, with strains BS64 (pBS64) and BS64 (pHB44) showing about 3-fold and 2-fold enhanced biofilm formation compared to plasmid-less BS64 ($F = 109.3$, $P < 0.001$) (Fig. 2C).

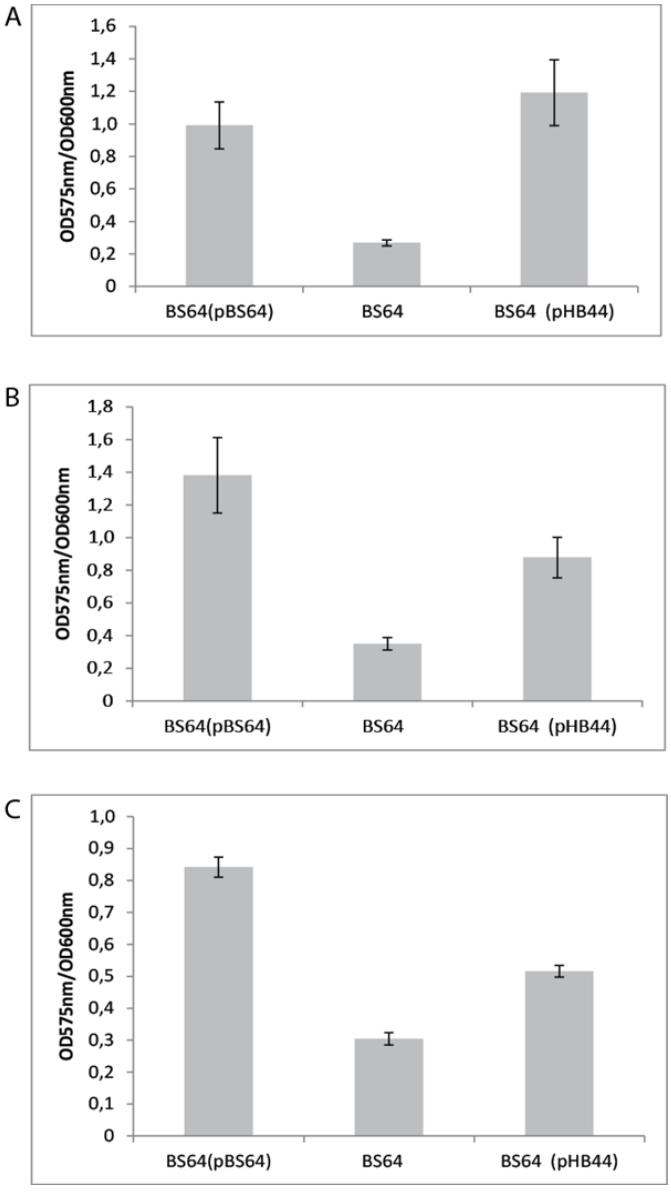

Fig. 2 Effect of plasmids pHB44 and pBS64 on biofilm formation by *V. paradoxus* strain BS64. The y-axes show the ratio OD575 nm/ OD600 nm, indicating the "normalized" amount of biofilm formation (see "Materials and Methods"). a 12 h; b 18 h; c 24 h

Effect of plasmids pHB44 and pBS64 on strain BS64 population dynamics in L. sp. strain Karsten affected liquid medium

L9+propionate medium successfully supported the growth of *L.* sp. strain Karsten (Boersma et al., 2010). Following growth, the fungal cells were removed and the medium filtered to obtain sterilized supernatant. Strains BS64 (pBS64), BS64 (pHB44) and plasmid-less strain BS64 - after their introduction - grew steadily in the *L.* sp. strain Karsten spent medium, from ~10^6 cells / ml to ~10^8 cells / ml over up to 9 d. No statistical differences were noted in the maximum population levels ($P > 0.05$). However, although not significantly different, at d.7, strain BS64 (pBS64) had established population sizes that were larger than those of the other two populations.

The population dynamics of *V. paradoxus* BS64 (pBS64) was then tested versus that of plasmid-less strain BS64 in liquid microcosms into which *L.* sp. strain Karsten had been allowed to grow and develop a fungal mat at the liquid-air interface (see [Nazir et al., 2013]). Overall, the dynamics of the two strains was similar, in that both slowly increased their densities from initial levels of about 10^5 CFU/ml to $9x10^5$ to $5x10^6$ CFU/ml over 28 days, after which they declined slowly, to about the initial levels at d.60. Strain BS64 (pBS64) ephemerally showed higher numbers than plasmid-less strain BS64 (at days14 and 28, respectively) (data not shown). Finally, an assessment of the numbers of buds and spikes on the fungal mats revealed statistically similar effects of the plasmid-containing and the plasmid-less strain. The average numbers of buds and spikes were, respectively, 40 and 5 for both strains after 60 days (see [Nazir et al., 2013]).

Effects of plasmid carriage on the dynamics of V. paradoxus strain BS64 in soil with L. sp. strain Karsten, in relation to Fe levels

To address the effects of plasmids pBS64 and pHB44 on host strain dynamics in a competitive situation for Fe with *L.* sp. strain Karsten in the G soil, the behavior of strains BS64 (pBS64), BS64 (pHB44) and the plasmid-less strain BS64 was tested in soil microcosms (Fig. 3A and 3B). None of the strains (inoculum levels ~10^5 CFU per g soil) survived in the soil without the fungal inoculum after 3 d (not shown). Previous work had already shown that *L.* sp. strain Karsten can rescue soil bacteria into the mycosphere (Nazir et al., 2012). Indeed, the experiments with the soil-invading *L.* sp. strain Karsten showed an outgrowth of all three strain BS64 types that had been introduced at the hyphal growth front.

In the Fe-unamended G soil after 6 d, a significantly higher bacterial densities were found for strains BS64 (pBS64) and BS64 (pHB44) as compared to the plasmid-less strain BS64 ($F = 11.49$, $P < 0.05$; Fig. 4A). Although this effect was already detectable at d 3, at the other time points there were no significant differences ($P>0.05$) between the CFU densities of the strains with and without the plasmids. Overall, the maximum population densities were reached after 11 d, being around 5 x10^7 CFU per g soil for all bacterial types (Fig. 3A).

We then assessed the population dynamics of the three aforementioned strains, at the fungal host, in the soil amended with $FeCl_3$ (Fig. 3B). This dynamics was largely similar across all strains, with a progressive increase of CFU densities and maximal densities being reached after 11 d for all strains. Remarkably, the density of strain BS64 (pBS64) increased at a faster pace than that of BS64 (pHB44) or plasmid-less strain BS64. In particular, strain BS64 (pHB44) revealed initially lowered CFU densities ($F = 38.57$, $P < 0.01$; Fig. 4B). Thus, under conditions of plentiful Fe supply, plasmid pBS64 enhanced the fitness of strain BS64, whereas, in contrast, plasmid pHB44 might impose a metabolic burden upon strain BS64 under these conditions.

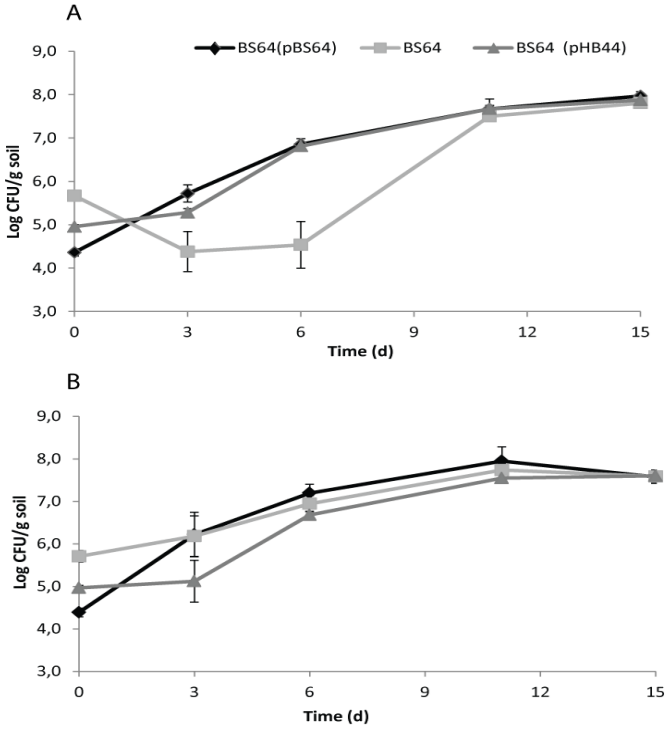

Fig. 3 Effect of carriage of plasmids pHB44 and pBS64 on the dynamics of *V. paradoxus* BS64 in *Lyophyllum* sp. strain Karsten-infested G soil microcosms. Separate inoculations. A. Native (unsupplemented) soil. B. Soil supplemented with 5 μM of $FeCl_3$

Competition for Fe^{3+} between plasmid-containing and plasmid-less V. paradoxus strains in soil containing growing L. sp. strain Karsten

The results of the joint inoculation experiments (Figs. 4 and 5) revealed remarkably different behaviors of the competing pairs as related to Fe levels. First, following initial declines, the population densities of strains BS64 (pBS64) and plasmid-less BS64 grew back to similar extents, establishing final densities of about 10^8 CFU per g dry soil in both Fe-amended and -unamended soils (Fig. 4A and 4B). In the soil with added Fe, plasmid-less strain BS64 showed higher CFU densities than BS64 (pBS64) on two out of five occasions (d 3: $F = 7.44$, $P < 0.05$; d 13: $F = 19.33$, $P < 0.05$). However, in Fe-unamended soil, the dynamics of both strains was indistinguishable, with strain BS64 (pBS64) being the better survivor at three of five time points. None of these differences was significant at any point. Thus, although the population dynamics of both strains was largely similar, a trend towards differential behavior of the two strains in microcosms with or without added Fe was discerned.

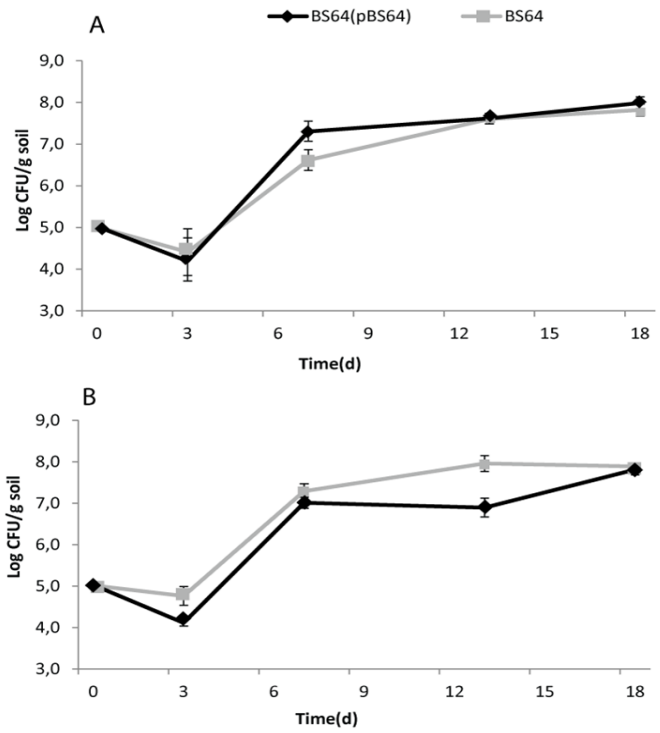

Fig. 4 Population dynamics of *V. paradoxus* BS64 (pBS64) versus plasmid-less BS64 in G soil microcosms colonized by *Lyophyllum* sp.strain Karsten. Joint inoculations. A Native (unsupplemented) soil, B Soil supplemented with 5 μM of $FeCl_3$

The strain BS64 (pHB44) / BS64 competing pair showed remarkable behavior (Fig. 5 A and B). In the soil with added Fe (Fig. 5B), plasmid-less strain BS64 slightly outperformed strain BS64 (pHB44). In contrast, the behavior in the soil with native Fe levels was very different. Strain BS64 (pHB44), at all occasions, strongly outperformed the plasmid-less counterpart ($F = 4.68$, $P < 0.05$). As from day 7, the significant differences were amounting to 1.5 to 2 log units in terms of CFU densities ($F = 19.71$, $P < 0.001$).

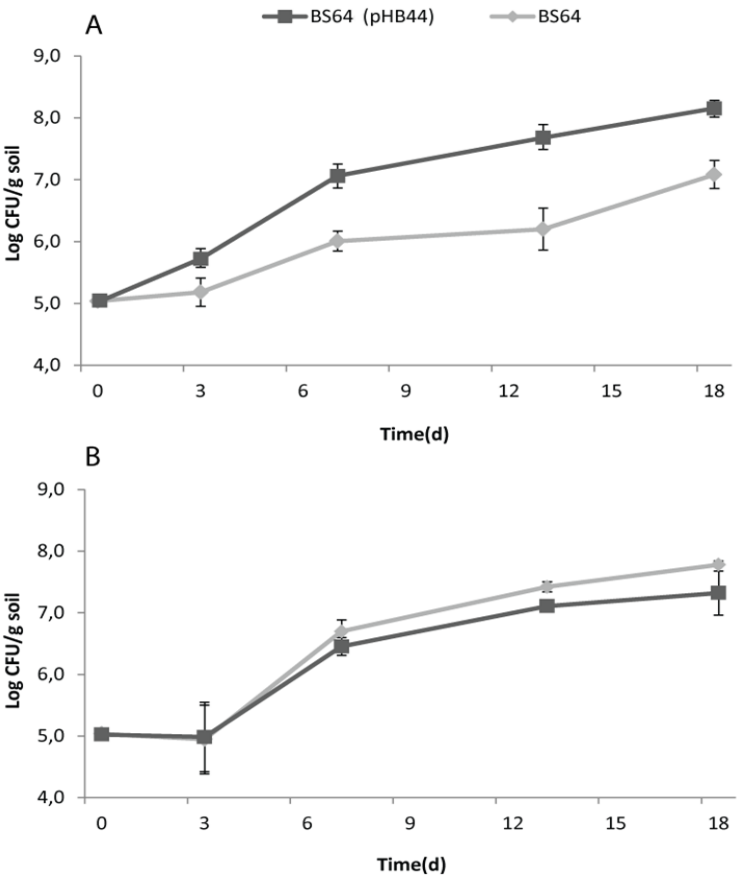

Fig. 5 Population dynamics of *V. paradoxus* BS64 (pHB44) versus plasmid-less BS64 in G soil microcosms colonized by *Lyophyllum* sp. strain Karsten. Joint inoculations. A. Native (unsupplemented) soil; B. Soil supplemented with 5 μM of $FeCl_3$

Discussion

In previous work on mycosphere-attracted bacteria, *Variovorax paradoxus* was among the most dominant culturable bacterial groups in the mycosphere of the EM fungus *Laccaria proxima*, whereas this organism was a minority isolate from corresponding bulk soil (Boersma et al., 2010). *Variovorax* spp. have indeed been often found in soil environments

(Boersma et al., 2010; Wang and Gu, 2006) and key roles in biogeochemical cycles (in particular steps of the carbon and sulfur cycles in soil) have been suggested for several species of this genus. Thus, involvement in the mineralization of particular herbicides has been found (Sorensen et al., 2009), with a particular *Variovorax* strain, SRS16, able to mineralize the commonly-used herbicide linuron.

The initial evidence for the presence of a type IV secretion system in the mycosphere-dwelling *V. paradoxus* strain HB44 (Boersma, 2010) was revealing, but it was inconclusive with respect to the underlying genetic system. We thus investigated a broader range, i.e. a total of fifteen *V. paradoxus* mycosphere-dwelling strains, and found that two of these, strains HB44 and BS64, indeed contained plasmids with sizes below roughly 100 Kb, one each per strain. The fact that not all strains were plasmid-endowed suggested that such plasmids represent rather 'flexible' genetic elements that may provide adaptive value in particular ecological conditions, but not in others, when they are counterselected as a result of the energetic burden posed upon the cell (Thomas, 2000). Another line of thinking poses that plasmids are ultimate genetic parasites (genetic 'ticks') that make use of their organismal hosts and move among them using their transfer apparatus. In any case, the finding of the two plasmids in a typical mycosphere population provides a possible clue as to the relevance of (some) plasmids as enhancers of the competence of their hosts in the mycosphere. The analyses of *trf*A gene sequences indicated both novel plasmids to most likely belong to the IncP-1β class. Indeed, the preliminary pHB44 backbone sequence analysis clearly revealed this plasmid to be most closely related to the typical IncP-1β plasmid R751 (Table 2). Some hints of a possible involvement of plasmid pHB44 in uptake processes taking place at the membrane, in particular of Fe, was found (Table 2), and so Fe was singled out as a possible ecological factor of interest. However, we have not yet obtained clear data about the complete accessory gene clusters, which would allow us to assign broader, albeit genetically-based, putative ecological roles.

On the basis of the foregoing data and assumptions, we formulated the hypothesis that carriage of IncP-1β plasmids, such as the ones found by us, might offer an ecological advantage to *V. paradoxus* mycosphere dwellers under particular, potentially ephemeral, conditions. Hence, a quest for plasmid-associated phenotypes was unleashed. The most straightforward way to disentangle the effects of plasmid-encoded from those of chromosomally-encoded traits is comparative, i.e. by comparing the behavior/activity of an organism containing a plasmid with that of an isogenic one devoid of that plasmid. We

therefore successfully cured *V. paradoxus* strain BS64 of its plasmid pBS64, yielding an important reference strain, BS64. The absence of plasmid pBS64 from this strain was confirmed by plasmid extraction as well as by PCR-based analysis of the *trf*A gene. Testing of growth rates in LB broth revealed broadly similar rates between the plasmid-cured and the plasmid-containing strain. In contrast, we were, for reasons yet to be understood, unable to produce a pHB44-cured version of strain HB44. However, plasmid pHB44 was successfully transferred from *V. paradoxus* HB44 to strain BS64, and thus a nice suite of strains was obtained to do phenotype assessments with. Quite remarkably with respect to antibiotic / heavy metal resistances and xenobiotic-degrading capacities, we could not discern any clear plasmid-associated phenotype conferred upon strain BS64 by any of the two IncP-1β plasmids (Table 3). This failure to detect any of these laboratory-detectable phenotypes appears to point to the fact that the putative selective pressures that the hosts of these plasmids are confronted with in their native setting (soil and the mycosphere) are likely to be different from the ones faced by hosts of other common IncP-1β plasmids (which often are carriers of such traits and are mostly obtained from polluted or antibiotic-containing habitats (Heuer et al., 2004; Schluter et al., 2007).

Table 3 – Summary of assessments of phenotypes conferred by plasmid pHB44 and pBS64 upon *V. paradoxus* BS64

Phenotype / effect on behavior	pHB44	pBS64	Remarks / method used
Antibiotic resistances*	-	-	None of common resistance found on IncP-1 type plasmids
Heavy metal resistances#	-	-	No typical IncP-1 plasmid resistances (e.g. Hg^r) found
Degradation of xenobiotics^	-	-	No typical IncP-1 plasmid-borne biodegradative capacities found
Stimulus of biofilm formation	+	+	Microtiter plate method used in (Djordjevic et al., 2002)
Increased survival in the mycosphere	+	+/-	Conditions of Fe scarcity
Decreased survival in the mycosphere	+	-	Conditions of plentiful Fe
Enhanced competitiveness in the acquisition of Fe	+	-	Hypothesis from soil experiments
Effect on fungal primordium setting	ND	-	Performed as in Nazir et al (2013)

-: no effect or phenotype found. +: significant effect of plasmid (in *V. paradoxus* BS64) found. +/-: potential effect found. ND: not determined.

*Antibiotics: ampicillin (Ap), kanamycin (Km), chloramphenicol (Cm), nalidixic acid (Nx), streptomycin (Sm), colistin sulphate (CS), nitrofurantoin (Nf), tetracycline (Tc), penicillin (Pc), spectinomycin (Sp), vancomycin (Vm),ergebicillin (Eb), erythromycin (Em) at concentrations 0, 5, 10, 20, 50, 100 μg/ml.

#Heavy metals: $HgCl_2$, $CuSO_4$, $NiCl_2$, $CoCl_2$, $ZnSO_4$, $CdCl_2$.

^Xenobiotic compounds: 2-chlorobenzoic acid, 3-chlorobenzoic acid, 4-chlorobenzoic acid, chlorocatechol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenoxy acetic acid, chloroacetate (sodium form), salicylate, toluene, xylene, 4-methyl toluene sulfonate.

In contrast, some striking effects of both plasmids carried by *V. paradoxus* BS64 were detected, and these were related to the ecological (fitness-enhancing) role of such plasmids. The first major finding was the significant increase of the amount of biofilm material produced by both plasmid-endowed versus -unendowed strains. This significant raise of biofilm biomass is consistent with previously-found effects by other plasmids (Ghigo, 2001). Thus Ghigo (2001) already demonstrated that derepressed conjugative plasmids, including plasmids of the IncN, IncW and IncP-1α groups, have a stimulatory effect on *E. coli* K12 biofilm formation. Moreover, the carriage of the IncP-9 group (TOL) plasmid pWWO can enhance biofilm formation in *Pseudomonas putida* KT2440 (D'Alvise et al., 2010), and similar results in this organism were observed for the IncP-1ε plasmid pKJK5 (Roder et al., 2013). Our results add to this emerging evidence, highlighting a novel feature of the IncP-1 β group of plasmids. One may speculate about such an effect at the fungal surface, where biofilm formation has been suggested to be an important asset of successful mycosphere colonizers (Warmink, 2009). Specifically, contact between *Burkholderia terrae* BS001 and *Lyophyllum* sp. strain Karsten hyphae possibly incites the development of a biofilm surrounding the hyphae.

The second key finding relates to the presumed ecologically-relevant traits donated by the two plasmids to the *V. paradoxus* BS64 host and concerns the survival capability / competitiveness at the fungal host surface, i.e. in cells in direct interaction with the fungal mycelium. We clearly obtained evidence for the contention that the presence of both plasmids can exert survival-enhancing effects on the *V. paradoxus* BS64 host in this setting, in which there may be competition, for instance for available Fe. For plasmid pBS64, this effect was broadly related to survival / ecological competence in systems with the fungal host, not being strongly related with the level of $FeCl_3$ present. One may surmise that the enhanced capacity

to produce biofilm material, coupled or not to a capacity to better utilize fungal-produced compounds, stimulates the survival of the plasmid-endowed bacterium at the fungal host surface. Remarkably, for plasmid pHB44, the story was different, as we obtained clear evidence for the contention that the *V. paradoxus* BS64 host endowed with this plasmid was better able to cope with the Fe-limiting conditions in the mycosphere than the plasmid-less BS64 strain. This was first noted in the separate-inoculation systems, and was later confirmed in the mixed-inoculation systems. Clearly, the effect was only detectable in the low-$FeCl_3$ condition, i.e. in the soil with native Fe level, whereas it was not detectable at high $FeCl_3$ levels. One may posit that (1) there was Fe scarcity in the native soil and (2) competition for Fe between strain BS64 and its fungal host took place. The presence of plasmid pHB44 thus assisted the bacterium in its Fe acquisition in the mycosphere, especially so in the paired-strain system (Fig. 5). This story held for the native soil, with Fe scarcity, but broke down in the soil with plentiful Fe. In fact, in the latter case, the plasmid pHB44-endowed strain BS64 revealed poorer survival than its plasmid-less counterpart, indicating an overriding effect of the energy burden conferred by the plasmid upon the cell.

Thus, the presence of plasmids in *V. paradoxus* strains can be an asset in conditions at the fungal host surface when either a biofilm may provide extra shielding or adherence (holds for pHB44 and pBS64) or Fe has to be sequestered in competition with the fungal host (true for pHB44). One may further ask whether the presence of *V. paradoxus* – in connection to the presence or not of plasmids – affected the survival or population dynamics of the fungal host. In our experimental setup, no influence of any of the used bacterial strains on the growth and development of *Lyophyllum* sp. Karsten was observed (data not shown).

In conclusion, we here examined the potential ecological role of the first IncP-1β plasmids obtained from an ectomycorrhizal fungal mycosphere, i.e. pHB44 and pBS64 in *V. paradoxus* strains HB44 and BS64, respectively. The plasmids were transferable over a broad host range within the Proteobacteria, as expected, and represent vehicles of horizontal gene transfer in the mycosphere habitat, thus potentially accelerating evolution in this gene transfer arena (Zhang et al., 2014). This is consistent with existing knowledge on the role of IncP-1β plasmids in other habitats (Heuer and Smalla, 2012; Schluter et al., 2003). Plasmids of the IncP-1β class can thus add to the versatility of bacterial genomes in the mycosphere, an understudied gene transfer arena where bacteria adapt to the emerging local conditions, in particular endowing them with enhanced potential to evolve towards fitness increases (Zhang et al., 2014). On the other hand, fitness costs of plasmid carriage were also shown in this work,

and hence bacteria that make use of plasmid-borne traits in the mycosphere appear, in the face of local conditions, to walk on a 'tiny rope' between fitness-increasing and -decreasing forces.

Acknowledgements

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